Mutation Research 772 (2014) 20–24

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Potential genotoxic effects of low-intensity ultrasound on fibroblasts, evaluated with the cytokinesis-block micronucleus assay

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ARTICLE INFO

Article history:
Received 22 August 2013
Received in revised form 29 May 2014
Accepted 28 June 2014
Available online 25 July 2014

Keywords:
Micronucleus
CBMN assay
NIH-3T3
Ultrasound
Genotoxicity

ABSTRACT

Although medical ultrasound offers compelling opportunities to improve therapy in principle, progress in the field has been limited because of an insufficient understanding of the potential genotoxic and cytotoxic effects of ultrasound on biological systems. This paper is mainly focused on an in vitro study of effects with respect to genotoxicity and viability induced by 1- and 3-MHz medical ultrasound in murine fibroblasts (NIH-3T3) at low-intensity exposure (spatial peak temporal average intensity Ita < 0.1 W/cm^2). The NIH-3T3 cells constitute a well-characterized in vitro cell model in which a genotoxic effect can be predicted by means of a reliable and precise murine cytokinesis-block micronucleus assay. A statistically significant increase in the incidence of micronuclei was observed in sonicated 3T3 cells. In particular, the effects were more evident at 1 MHz. Moreover, for each frequency investigated, the occurrence of micronuclei was comparatively more frequent with increasing time of exposure. The possible toxicological implications of the medical ultrasound employed herein deal with the existence of a window of exposure parameters (set well below the intensity of ultrasound cavitation) in which some genotoxic effects may occur without significant cytotoxicity. In this respect, they provide new insight toward the correct risk to benefit balancing of ultrasound-based treatments and for designing innovative therapeutic strategies.

1. Introduction

During the past few years, biomedical acoustics have been rapidly evolving from a diagnostic modality into a therapeutic tool, alone or in combination with drugs. In particular, the technique has been applied at frequencies of ~1–3 MHz in the treatment of diabetes, stroke, cancer, cardiovascular diseases, infections, osteoporosis, thrombosis, glaucoma, nerve damage, skin wounds, and bone fractures [1–6]. Despite the availability of much evidence on beneficial properties of ultrasound for human health, progress in the field to date has been severely limited by insufficient understanding of tissue sensitivity to damage caused by ultrasound [1].

Depending on several exposure parameters – primarily frequency and intensity – the modes of interaction of ultrasonic waves with tissue are commonly broadly classified as "thermal" and "non-thermal" with respect to mechanical energy transfer [3]. In a fluid medium undergoing an ultrasonic field, this energy transfer is thought to be mediated by the cavitation activity, i.e. the oscillation and implosion of gas bubbles [4]. Ultrasonic cavitation has been considered as the cause of several destructive effects on plasma-membrane structure, chromosomes, mitotic figures, and nuclei [7–17]. It has been suggested that cavitation induces chromosome breakage through the action of residual hydrogen peroxide [7,18]. The induction of single-strand breaks in DNA was detected in vitro with a comet assay, after exposure of cell suspensions to 2.17-MHz ultrasound [18,19]. Nevertheless, to date, such a supposed correlation between ultrasonic cavitation and biological damage remains to be demonstrated [10,20].

Ultrasonic exposure under low-intensity, non-cavitation regimes, when the spatial peak temporal average intensity Ita remains below 100 mW/cm^2, has in fact also been reported to produce significant biological effects, such as membrane deformation, increased cell permeability, and chromosomal alteration

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http://dx.doi.org/10.1016/j.mrgentox.2014.07.004
1383-5718/

[17,20–23]. In the latter framework, according to the recent bilayer-sonophore model, alterations in structure and permeability of cell-plasma membranes can occur when the elastic tension of the bilayer leaflets undergoing ultrasonic pressure exceeds that required for the opening of membrane pores [20].

In contrast with the studies on bilayer plasma-membranes, there is little evidence on the genotoxic and cytotoxic potential associated with mechanical insult under low-intensity sub-cavitation ultrasound regimes. Existing reports present conflicting results depending on the test system applied [17]. Studies have reported the effects of medical ultrasound on DNA integrity [21], and on survival, colony formation, and attachment of cultured cells [22]. However, several authors detected no chromosome damage in sonicated lymphocytes and fibroblasts [23–25]. Nevertheless, the lack of visible chromosomal damage does not exclude the possibility of damage to the DNA molecules. Indeed, ultrasound is routinely used to break-up purified DNA molecules [26,27].

Among assays used for assessment of genomic damage, the in vitro micronucleus test has become an attractive tool because of its simplicity of scoring, accuracy, and wide applicability [28–32]. The evolution of the micronucleus test toward assessment of multi-target genotoxic endpoints and cancer prediction through the development of the cytokinesis-block technique allows us to overcome the confounding influence on micronucleus formation from the cytostatic effects caused by poor culture conditions, effects of the treatment, cell senescence, and variability in mitogen response [32–34]. For these reasons, it can successfully be employed in a robust quantitative extrapolation to define potential limits or thresholds of exposure [35].

In this framework, we have previously applied the well-established murine cytokinesis-block micronucleus (CBMN) protocol for assessment of a genotoxic risk in NIH-3T3 cell lines exposed to low-intensity ultrasound. Unexpectedly, at 1 MHz and Ita ~28 mW/cm^2 (i.e. below the cavitation threshold), a statistically significant increase in the frequency of micronuclei was observed [36]. Based on these preliminary results, in the present study we exploited the in vitro CBMN assay and viability tests to analyze genotoxic and cytotoxic effects of ultrasound on cultured murine fibroblasts (NIH-3T3) in the absence of cavitation, and to determine the cellular response to a therapeutically relevant duration of exposure to ultrasound frequencies of 1 and 3 MHz.

2. Material and methods

2.1. Cell line and culture conditions

The experiments were performed with NIH-3T3 murine fibroblasts (Sigma–Aldrich, St. Louis, MO, USA). The cells were cultured in a humidified atmosphere with 95% air and 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (Sigma–Aldrich) with 10% fetal bovine serum (Sigma–Aldrich), 1% L-glutamine (Sigma–Aldrich), and 1% penicillin/streptomycin (Sigma–Aldrich). Fibroblasts were plated in well plates (Falcon® Easy GripTM tissue-culture dishes, 35 × 10 mm, Franklin Lakes, NJ, USA) containing 3 mL of media and grown for about 48 h to 90% confluence (~7–9 × 10^5 cells per plate); the cells were then sonicated as described in Section 2.2.

2.2. Ultrasound exposure-system and experimental protocol

For the exposures to ultrasound, two conventional medical devices (Nuova Elettronica, BO, Italy) were used consisting of two submersible piezo-ceramic circular transducers (6-cm diameter) tuned at 1 and 3 MHz (maximum nominal intensity = 2.5 W/cm^2) with their generators. Such systems operate in the range from 10 to 100% of the maximum power in continuous and pulsed mode. In pulsed mode, the duty cycle can be selected; in high mode, the signal is delivered for 750 ms followed by a pause of 250 ms. For both 1- and 3-MHz ultrasound exposures, the pulsed mode was used in "high".

Ultrasonic transducers were placed at the bottom of a tank (30 × 30 × 30 cm) filled with degassed water. A hermetically closed Petri dish (9.6 cm^2) containing the adherent cells was positioned at the water surface and submerged up to half its thickness, in line with the transducer [36]. The temperature of the bath was kept constant at 25 °C (accuracy, 1 °C) both inside and outside the Petri dish.

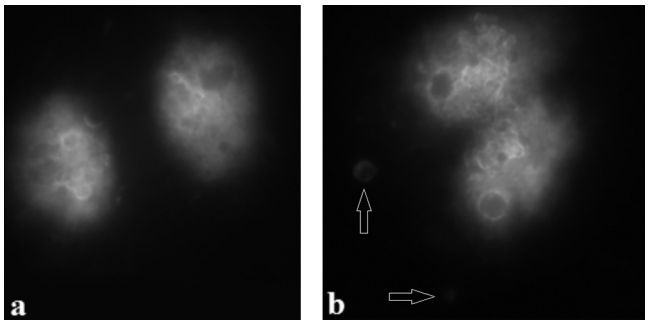

Fig. 1. Fluorescence image (1000× magnification) showing bi-nucleated cells (a) and bi-nucleated cells containing micronuclei (b), stained for nuclear DNA with DAPI; micronuclei are indicated by arrows.

The acoustic fields produced by the ultrasound sources at the two frequencies were characterized. This consisted of measuring the ultrasonic field intensity varying the distance between the ultrasound source and the lower surface of the plate by means of a hydrophone needle (S.N. 1470, Precision Acoustics, UK) of 1 mm diameter with sensitivities of 1670.4 mV/MPa and 958.2 mV/MPa (±14%) at 1 MHz and 3 MHz, respectively.

The biological samples were sonicated at a 5-cm distance (at which the ultrasonic field was evaluated as stable and reproducible) in "high" mode for exposure times of 5, 15, 30, 45, and 60 min according to therapeutic protocols.

In this study, the intensity of the acoustic field was provided in terms of 'spatial peak temporal average intensity' (Ita), which represents the maximum spatial intensity measured when the pulse is on, mediated for the period of pulse repetition. It is commonly used to correlate biological effects induced by low-intensity ultrasound [20]. The overall Ita values observed were 7.1, 11.8, 15.2, and 19.3 mW/cm^2 for the 1-MHz exposure, and 1.0, 4.9, and 7.0 mW/cm^2 for the 3-MHz exposure set-up; all of these values are below the 100-mW/cm^2 cavitation threshold [20].

2.3. Micronucleus test

The control and sonicated cellular samples (eight samples for each measurement) were treated with 6 μg/mL of cytochalasin B (Sigma–Aldrich) immediately after sonication and then grown for 24 h to allow accumulation of bi-nucleated cells. The cells were then collected by centrifugation, suspended in a pre-warmed hypotonic solution (75 mM KCl), and fixed in Carnoy's fixative (acetic acid/methanol, 1:3, v/v). Air-dried samples, prepared by dripping the fixed cells onto pre-cleaned slides, were stained with 10 mg/mL of 4′-6′-diamidino-2-phenylindole (DAPI, Sigma–Aldrich) in anti-fade solution (Vector Laboratories, Burlingame, CA, USA). Micronuclei were scored at 1000 × magnification with a Zeiss Axiophot microscope (Zeiss Micro-imaging Inc., Thornwood, NY, USA) with ultraviolet light (with a 359-nm excitation filter and a 441-nm barrier filter). The samples were coded and scored blindly by the same analyst. A representative fluorescence image of DAPI-stained, bi-nucleated cells and bi-nucleated cells containing micronuclei is shown in Fig. 1a and b, respectively. For all eight replicates of each sample, 500 bi-nucleated cells were scored [31].

2.4. Cell-viability test

Cell viability was determined for eight cell-culture plates for both the untreated (control) and the ultrasound-treated (sonicated) cell samples by use of the Trypan-blue exclusion test.

Cell viability before exposure to ultrasound was greater than 95% for each trial. Recently, a close correlation has been suggested between the lack of wild-type permeability and mechanical stress that occurs at the plasma-membrane level of 3T3 cells exposed to low-intensity ultrasound [20,37]. For this reason, the viability tests were started 10 min after the end of sonication to allow the structural integrity of the plasma membrane to recover [37,38].

2.5. Data analysis

Both the frequency of micro-nucleated cells and the viability data for treated cells and untreated controls were expressed as mean values ± standard deviation from eight independent experiments. A one-tailed Dunnett's test was used to evaluate the statistical significance of differences between treated and untreated samples for genotoxicity and viability. Inter-group comparisons between samples treated with different intensities and for different time periods were assessed via analysis of variance (ANOVA) followed by the Student's *t*-test. The level of significance was established at $p < 0.05$.